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(54) Title: VEGF-B/RECEPTOR COMPLEX AND USES THEREOF			
(57) Abstract <p>Disclosed are complexes of Vascular Endothelial Growth Factor-B (VEGF-B) and the Flt-1 tyrosine kinase receptor; the use of such VEGF-B/Flt-1 complexes in assays for VEGF-B or for VEGF-B analogs characterized as having substantially the same binding affinity for a cell surface receptor as VEGF-B, and/or in promoting or antagonizing a VEGF-B-mediated cell response; and specific binding partners, e.g. antibodies, for such VEGF-B/Flt-1 complexes.</p>			

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VEGF-B/RECEPTOR COMPLEX AND USES THEREOFBackground of the Invention

The present invention relates to a complex of Vascular Endothelial Growth Factor-B (VEGF-B) and the Flt-1 receptor, to methods of using such complexes to induce or antagonize a VEGF-B-mediated cellular response, to assay kits for identifying VEGF-B and/or VEGF-B analogs, and to isolated binding partners, such as antibodies, which bind to VEGF-B/Flt-1 complexes.

Vascular Endothelial Growth Factor (VEGF or VEGF-A; sometimes also referred to as Vascular Permeability Factor or VPF) is an angiogenic growth factor of the PDGF family. It exerts its effect through two endothelial receptor tyrosine kinases (RTKs), Flt-1 (also known as VEGFR-1) [Shibuya et al., *Oncogene*, 5:519-524 (1990); de Vries et al., *Science*, 255:989-991 (1992)] and Flk-1/KDR (also known as VEGFR-2) [Matthews et al., *Proc. Natl. Acad. Sci. USA*, 88:9026-30 (1991); Terman et al., *Biochem. Biophys. Res. Comm.*, 187:1579-86 (1992); Millauer et al., *Cell*, 72:835-46 (1993)]. These receptors appear to play a pivotal role in regulation of endothelial cell growth and differentiation and in maintenance of the functions of the mature endothelium [Shalaby et al., *Nature*, 376:62-66 (1995); Fong et al., *Nature*, 376:66-70 (1995)]. VEGF and its high affinity receptors Flt-1 and KDR/flk-1 are required for the formation and maintenance of the vascular system as well as for both physiological and pathological angiogenesis.

Placenta growth factor (PlGF) [Maglione et al., *Proc. Natl. Acad. Sci. USA*, 88:9267-71 (1991)] is another ligand for the Flt-1 RTK [Park et al., *J. Biol. Chem.*, 269:25646-54

(1994)]. It is also a member of the PDGF family and is structurally related to VEGF, but its biological function is not presently well understood.

5 VEGF-B is a distinct growth factor for endothelial cells described in Olofsson et al., "Vascular endothelial growth factor B; a novel growth factor for endothelial cells", *Proc. Natl. Acad. Sci. USA*, 93:2576-81 (1996). Like VEGF and PlGF, it is a member of the PDGF family of growth factors with which it shares substantial structural
10 similarities, including a pattern of conserved cysteine residues which form disulfide bonds involved in homo- and hetero-dimerization of the molecule. Nevertheless, VEGF-B exhibits only approximately 40 to 45 percent sequence similarity to VEGF and only approximately 30 percent
15 sequence similarity to PlGF. VEGF-B has been found to be co-expressed with VEGF in various tissues and is particularly abundant in heart and skeletal muscle tissue. It promotes mitosis and proliferation of endothelial cells and appears to have a role in endothelial tissue growth and
20 angiogenesis. VEGF-B may potentiate the mitogenic activity of low concentrations of VEGF both *in vitro* and *in vivo*.

The present invention is based on the discovery that VEGF-B is capable of binding to the extracellular domain of Flt-1 receptor tyrosine kinase to form bioactive complexes
25 which mediate useful cell responses and/or antagonize undesired biological activities.

References herein to the amino acid sequence of VEGF-B refer to the sequence for human VEGF-B₁₈₆ described in Eriksson et al., published PCT Application No. WO 96/26736
30 (Genbank database accession no. U52819). References to the amino acid or nucleotide sequences of Flt-1 refer to the sequences described by Shibuya et al., *Oncogene*, 5:519 (1990), (EMBL database accession no. X51602). Binding affinity of VEGF-B and/or VEGF-B analogs for the Flt-1
35 receptor or analogs thereof is tested according to the procedure described in Lee et al., *Proc. Natl. Acad. Sci.*,

93:1988 (1996). A useful method for assaying endothelial cell proliferation is described in Olofsson et al., *Proc. Natl. Acad. Sci. USA*, 93:2576-81 (1996).

5 In accordance with one preferred aspect of the invention, the invention relates to a method for identifying a VEGF-B analog having substantially the same binding affinity for a cell surface receptor as VEGF-B, the method comprising the steps of:

10 (a) providing a sample containing a receptor protein selected from the group consisting of:

(i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
15 (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; and

(iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that
20 hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;

(b) contacting said sample of step (a) with a candidate VEGF-B analog; and

25 (c) detecting specific binding between the candidate VEGF-B analog and the receptor protein of step (a).

30 VEGF-B binding to a cell surface receptor is considered to involve a VEGF-B dimer binding to the receptor which causes dimerization of the receptor and autophosphorylation of that receptor followed by intra-cellular signalling and, in appropriate circumstances, a cellular response such as angiogenesis.

35 The sample containing receptor protein could be, for example, soluble Flt receptor produced naturally in the

conditioned medium of cells that normally express the receptor. Tissue samples or tissue fluids shed naturally from cells by proteolytic events also could be used as receptor samples.

5 The VEGF-B analogs identified by this aspect of the invention may be small molecules, for example proteins or peptides or non-proteinaceous compounds such as DNA or RNA. The analogs also could include VEGF-B or a derivative (including, but not limited to, a fragement of a VEGF-B monomer or dimer) tagged with a toxin or drug or radioactive
10 isotope which could target Flt-1 expressed and upregulated on endothelial cells in tumors. Such molecules could be useful to antagonize or inhibit unwanted VEGF-B induced cellular responses such as tumor-induced angiogenesis or psoriasis or retinopathies by techniques analogous to those
15 described in Kim et al., *Nature*, 362(6243):841-44 (1993) or Aiello et al., *New England Journal of Medicine*, 331(22):1480-87 (1994).

One procedure for isolating VEGF-B/Flt-1 complexes
20 involves using fusion proteins of the Flt-1 receptor and immunoglobulin G (IgG) followed by Sepharose A binding. Alternatives to the use of Sepharose A include using ion-exchange chromatography, gel filtration or affinity chromatography. Conditioned medium containing receptor/IgG
25 fusion proteins could be allowed to interact with conditioned medium either from cells either transfected with DNA encoding the VEGF-B ligand or analog thereof, or from cells which naturally express the ligand, or with a solution containing a candidate ligand analog.

30 In accordance with another preferred aspect of the invention, the invention relates to a method for identifying a VEGF-B analog having substantially the same binding affinity for a cell surface receptor as VEGF-B, the method comprising the steps of:

(a) providing a sample containing cells that express a surface receptor protein having binding affinity for VEGF-B selected from the group consisting of:

- 5 (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1;
- 10 and
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined
- 15 by nucleotides 1-1293 of Flt-1;

(b) contacting the cells with a candidate VEGF-B analog, and

(c) detecting induction of a VEGF-B-mediated cellular response. Examples of such detectable cellular responses

20 include endothelial cell proliferation, angiogenesis, tyrosine phosphorylation of receptors, and cell migration.

The cells which express the cell surface receptor protein may be cells which naturally express the receptor, or they may be cells transfected with the receptor such that

25 the receptor is expressed. Conditioned medium from culturing such cells can be passed over a Sepharose A column or matrix to immobilize the receptor, or they can be immobilized in cellulose disks or absorbed onto plastic, in the form of an ELISA test. A second solution containing

30 conditioned medium from cells expressing the ligand is then passed over such immobilized receptor. If desired, the ligand may be radioactively labelled in order to facilitate measurement of the amount of bound ligand by radioassay techniques. Such an assay can be used to screen for

35 conditions involving overexpression of the Flt-1 receptor, i.e. through detection of increased bound radioactivity

compared to a control. This methodology can also be used to screen for the presence of competing VEGF-B analogs, i.e. through detection of decreased bound radioactivity compared to a control indicative of competition between the radioactively labelled VEGF-B ligand used in the test and a non-radioactive putative analog.

Alternatively, the foregoing assay could be reversed by immobilizing the VEGF-B ligand or candidate analog and contacting the immobilized ligand with conditioned medium from cells expressing the receptor.

In accordance with yet another aspect of the invention, the invention relates to a kit for identifying VEGF-B or a candidate VEGF-B analog in a sample, the kit comprising:

(a) a receptacle adapted to receive a sample and containing a receptor protein selected from the group consisting of:

- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; and
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;

and

(b) means for detecting interaction of VEGF-B or a candidate VEGF-B analog with the receptor protein contained in the receptacle, wherein the VEGF-B or candidate VEGF-B analog comprises part of a sample received in the receptacle. The detecting means may comprise, for example, means for detecting specific binding interaction of VEGF-B or a VEGF-B analog with the receptor protein or means for detecting induction of a VEGF-B induced cellular response.

A still further aspect of the invention relates to an isolated ligand-receptor complex comprising two molecules, one defining the ligand and comprising at least amino acids 1-115 of VEGF-B or a receptor-binding analog thereof, and the second defining the receptor and being selected from the group consisting of:

- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- 10 (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1;
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that
15 hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1.

Preferably the ligand is VEGF-B and the receptor is the Flt-1 receptor which also has binding affinity for VEGF-A and PlGF.

Isolation and purification of the ligands or complexes could be effected by conventional procedures such as immunoaffinity purification using monoclonal antibodies according to techniques described in standard reference works such as Harlow et al., *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory Press (1988) and/or Marshak et al., *Strategies for Protein Purification and Characterization*, Cold Spring Harbor Laboratory Press (1996). Suitable antibodies to the individual ligands or to the complexes could be generated by conventional techniques.

A cell-free complex could be used either *in vivo* or *in vitro* to compete with VEGF-B binding to a cell surface receptor or to prevent dimerization of the cell-bound receptor after ligand binding. Such a cell-free complex would comprise at least one receptor molecule, for example soluble FLT (sFLT), and a VEGF-B dimer molecule, VEGF-B analog dimer molecule or mixed VEGF-B/VEGF-B analog dimer

molecule so that one molecule of the dimer can be bound to the receptor molecule in the complex and the second molecule of the dimer has a free binding site available to bind to a cell surface receptor.

5 It is also an aspect of the present invention to provide an isolated binding partner having specific binding affinity for an epitope on a ligand-receptor complex comprising VEGF-B protein or an analog thereof in specific binding interaction with the ligand binding domain of a cell
10 surface receptor defined by:

- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for
15 VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; or
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a
20 nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;

wherein the binding partner has substantially no binding affinity for uncomplexed VEGF-B or VEGF-B analog. Preferably the binding partner also will have substantially
25 no binding affinity for any uncomplexed form of the cell surface receptor protein or receptor analog thereof. The binding partner may be an antibody which reacts with or recognizes such growth factor/receptor complexes. Either polyclonal or monoclonal antibodies may be used, but
30 monoclonal antibodies are preferred. Such antibodies can be made by standard techniques, screening out those that bind to either receptor or ligand individually.

An additional aspect of the invention relates to the use of a VEGF-B analog obtained according to the methods
35 described above for

- (i) antagonizing VEGF-B binding to a cell surface receptor, or

- (ii) antagonizing induction of a VEGF-B-mediated cellular response.

A preferred VEGF-B analog comprises an antibody having binding specificity for

- 5 (i) the ligand binding domain of a cell surface receptor having binding affinity for VEGF-B, or
- (ii) a receptor binding domain of VEGF-B or a receptor-binding analog thereof.

The ligand binding domain of a cell surface receptor
10 having binding affinity for VEGF-B desirably will exhibit at least 30%, preferably at least 35%, amino acid identity with residues 1-347 of Flt-1 and especially preferably will correspond thereto. The receptor binding domain of a VEGF-B
15 analog desirably will exhibit at least 50%, preferably at least 65%, sequence identity with amino acid residues 1-115 of VEGF-B, and especially preferably will correspond thereto.

Yet another aspect of the invention relates to the use of a receptor protein selected from the group consisting of:

- 20 (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid
25 identity with residues 1-347 of Flt-1; or
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by
30 nucleotides 1-1293 of Flt-1;

in a method for antagonizing:

- (a) VEGF-B binding to a cell surface receptor, or
- (b) induction of a VEGF-B-mediated cellular response.

The polypeptide chain competes with the cell surface
35 receptor for VEGF-B and ties up the available VEGF-B, thereby preventing it from effectively interacting with the cell surface receptor and inducing the VEGF-B mediated

cellular response. A suitable peptide chain could be a solubilized form of the receptor (sFLT) as described in Kendall et al., *Proc. Natl. Acad. Sci.*, 90:10705-709 (1993).

5 Additionally, it is an aspect of the invention to provide a method for antagonizing VEGF-B binding to a cell surface receptor, the method comprising the step of providing a protein having binding specificity for the amino acid sequence defined by residues 1-347 of Flt-1 or a VEGF-B receptor binding sequence variant thereof, wherein the
10 protein has at least 50%, and preferably at least 65%, amino acid sequence identity with residues 1-115 of VEGF-B, such that the protein, when provided to a cell expressing the cell surface receptor, is competent to interact specifically with the receptor and thereby substantially inhibits VEGF-B
15 binding to the receptor. The protein may desirably be a VEGF-B analog obtained according to one of the methods described above.

In accordance with a further aspect of the invention, pharmaceutical preparations are provided which comprise such
20 growth factor/receptor complexes.

In yet another aspect of the invention a method is provided for treating a disease state characterized by overexpression of an Flt-1 cell surface receptor, said method comprising administering to a patient suffering from
25 said disease state an effective receptor-binding amount of VEGF-B or a VEGF-B analog obtained according to one of the methods described above.

Where the receptor protein comprises a polypeptide chain other than residues 1-347 of Flt-1 but which
30 nevertheless exhibits a binding affinity for VEGF-B, it should exhibit at least 30%, desirably at least 35%, preferably at least 65%, particularly preferably at least 90%, and especially preferably at least 95%, amino acid identity with residues 1-347 of Flt-1. Useful VEGF-B
35 analogs should exhibit at least 50%, preferably at least 65%, particularly preferably at least 90%, and especially preferably at least 95%, sequence identity to VEGF-B.

Brief Description of the Drawings

The invention will be described in further detail hereinafter with reference to illustrative experiments, the results of which are illustrated in the accompanying drawings in which:

5 Figure 1 is an anti-PTyr probed Western blot of Flt-1 immunoprecipitates from Flt-1 expressing NIH3T3 cells stimulated with conditioned media from 293 EBNA cells transfected respectively with expression vectors for human VEGF and VEGF-B;

10 Figures 2(a) and (b) are respectively long and short exposures of SDS-PAGE electrophoresis gels showing binding of ³⁵S-methionine-labelled murine VEGF-B₁₈₆ to Flt-1-IgFc fusion protein;

15 Figure 3 is an SDS-PAGE analysis of the binding of VEGF₁₆₅, VEGF-B₁₆₇, VEGF-B₁₈₆ and VEGF-C to soluble VEGFR-1, VEGFR-2 and VEGFR-3;

Figure 4 is a graph of the displacement of [¹²⁵I]-hVEGF₁₆₅ from VEGFR-1/Flt-1 by mVEGF-B₁₈₆ using NIH3T3 Flt-1 cells;

20 Figure 5 is a graph of competition on NIH-VEGFR-1/Flt-1 by mVEGF₁₆₄;

Figure 6 shows displacement of VEGF-B₁₆₇ and VEGF-B₁₈₆ from soluble VEGFR-1 by excess VEGF₁₆₅;

25 Figure 7 is an SDS-PAGE analysis showing proteolytic processing of VEGF-B₁₈₆;

Figure 8 is an SDS PAGE analysis showing plasmin digestion of VEGF-B₁₈₆;

30 Figure 9 is a schematic diagram showing mutations of VEGF-B₁₆₇ used for mutational analysis and binding of receptor binding epitope mutants to soluble VEGFR-1;

Figures 10a through 10c show an SDS-PAGE analysis of cysteine mutations of VEGF-B;

Figure 11 shows an SDS-PAGE analysis of VEGF-B₁₆₇ mutants labelled in the presence of 10 µg/ml heparin;

35 Figure 12 shows a Northern Blot analysis of RNA from bovine microvascular endothelial (BME) cells incubated in the presence of 50 ng/ml hVEGF-B₁₈₆; and

Figures 13a and 13b show a zymographic and reverse zymographic analysis of cell extracts prepared from BME cells incubated in the presence of hVEGF-B₁₈₆.

5

General Methods

Cell culture and materials:

Sf-9 cells were maintained in Sf-900 II SFM (Gibco BRL, Life Technologies) supplemented with 0.1% pluronic f-68 for suspension growth.

10

High Five cells (Invitrogen) were maintained in Ex-cell 400 media (JHR Bioscience UK).

15

293-EBNA, COS-7, 293-T and NIH3T3-Flt-1 cells [Sawano et al., *Cell Growth & Differentiation* 7, 213-21 (1996)] were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). NIH3T3 Flt-1 were kept under continuous selection using 200 µg/ml neomycin.

20

Bovine adrenal cortex-derived microvascular endothelial (BME) cells were grown in MEM, alpha modification (Gibco AG, Basel, Switzerland) supplemented with 15% donor calf serum on 1.5% gelatin coated tissue culture flasks.

25

PAE-KDR cells [Waltenberger et al., *J. Biol. Chem.*, 269:26988-95 (1994)] were cultured in Ham's F12 media with 10% FCS.

Construction of receptor Ig-fusions and Expression Vectors:

a) pIg-VEGFR-1.

The expression plasmid pIg-VEGFR-1 coding for the first five Ig-like domains of VEGFR-1 fused to human IgG1 Fc was constructed by ligating a HindIII fragment (coding for the amino acids 1-549 of VEGFR-1) from pLTR Flt1 into pIgplus vector (Ingenius). Prior to the cloning the pIgplus vector was digested with XhoI and XbaI, blunted and religated in order to correct the reading frame for the fusion protein production.

b) spIg-VEGFR-2.

For the spIg-VEGFR-2 construct, cDNA encoding the first four Ig-like domains of VEGFR-2 was amplified by polymerase chain reaction (PCR) using human fetal lung cDNA library
 5 (Clontech) as a template. The primers

5'-atggtacccccaggctcagcatacaaaaagac-3' (SEQ ID NO. 1)

and

5'-gcgtctagagggtgggacatacacaaccag-3' (SEQ ID NO. 2)
 were used, and the amplified fragment was cleaved with Kpn-1
 10 and Xba-1 and cloned into corresponding sites of signal pIg
 vector (Ingenius).

c) mVEGF-B₁₈₆ pFASTBAC1.

mVEGF-B₁₈₆ cDNA [Olofsson et al., *J. Biol. Chem.*
 15 271:19310-19317 (1996)] was cleaved by EcoRI and subcloned
 into pFASTBAC1 (Gibco BRL Life Technologies). A (His)₆ tag
 (and an enterokinase site) was introduced at the N-terminus
 devoid of signal sequence, using PCR with mVEGF-B₁₈₆ pSG5 as
 a template, and the primers

20 5'-atcgagatcttcatcaccatcaccatcacggagatgacgatgacaaacctgtgtc
 ccagttt-3' (SEQ ID NO. 3) and

5'-caagggcggggcttagagatctagct-3' (SEQ ID NO. 4)

(both containing Bgl II sites) were used. The amplified
 fragment was cleaved with Bgl II and cloned into the Bam HI
 25 site in frame with the signal sequence of GP-67, of
 pAcGP67A, (Pharmingen, U.S.A.).

d) hVEGF-B₁₈₆ pPIC-9.

hVEGF-B₁₈₆ was amplified by PCR using the forward primer
 30 5'-ggaattcccccggccaggccctgtc-3' (SEQ ID NO. 5)
 and the reverse primer

5'-ggaattcaatgatgatgatgatgatgagccccggccttggc-3' (SEQ ID NO.
 6).

The amplified product containing a C-terminal (His)₆ tag was
 35 cloned into the EcoRI site of pPIC-9 (Invitrogen) in frame
 with the alpha mating factor signal sequence.

The authenticity of all sequences was verified by sequencing.

Protein Expression and Purification:

5 For baculoviral production using Sf9 and High Five cells, mVEGF-B recombinant plaques were purified and amplified [Summers et al., *Tex. Agric. Exp. Stn. Bull.* 1555:1-57 (1988)], and the corresponding expressed proteins as well as the *Pichia pastoris* (strain GS115) expressed
10 hVEGF-B₁₈₆ were purified using Ni-NTA Superflow resin (Qiagen).

For quantitative immunoblots, media from infected insect cells were run together with 1-30 ng purified m(His)₆VEGF-B₁₈₆ as a standard on a reducing 12% SDS-PAGE and
15 blotted with the affinity purified antibody against m(His)₆VEGF-B₁₈₆.

Transfection, immunoprecipitation and soluble receptor binding:

20 293-T cells or COS-7 cells were transfected with hVEGF₁₆₅PSG5, mVEGF-B₁₆₇PSG5, mVEGF-B_{KEx1-5}PSG5, mVEGF-B₁₈₆PSG5, VEGFR-1 pIg and VEGFR-2 pIg using calcium phosphate precipitation. VEGFR-3 EC-Ig pREP7 (obtained from Dr. Katri Pajusola) and hVEGF-CANΔC (His)₆ pREP7 [Joukov et al., *EMBO J.* 16:3898-911 (1997)] were similarly expressed in 293-EBNA
25 cells. The cells expressing the growth factors were metabolically labelled 48 hours post transfection with 100 μCi/ml Promix TM L-35 S (Amersham) for 5-6 hours (unless otherwise stated), and the media were collected. Heparin (10
30 or 50 μg/ml) was added to the labeling medium when indicated. The metabolically labelled media (except from the VEGF transfection) was immuno-depleted of endogenous expressed VEGF and heterodimers for 2 hours with 2μg/ml VEGF antibody MAB 293 (R&D Systems). For the soluble receptors
35 the media was replaced 48 hours post transfection by DMEM containing 0.1% BSA and incubated for additional 12 hours.

The receptor-Ig fusions (in some cases the amounts were quantified on 10% SDS-PAGE stained with Colloidal Comassie (Novex, San Diego)) and the same volume of media from mock transfected cells were absorbed to protein-A-Sepharose. The
5 metabolically labelled growth factors were incubated with the receptor Ig fusions for 3 hours at + 4°C and washed with ice-cold binding buffer (PBS 0.5% BSA, 0.02% Tween 20 and 1mM PMSF) three times and twice with PBS containing 1mM PMSF.

10

Antibody Production:

Rabbits were immunized with purified m(His)₆VEGF-B₁₈₆ according to standard procedures and the resulting antiserum was collected. Antiserum to mVEGF-B N-terminal peptide was
15 produced as described in [Olofsson et al., *J. Biol. Chem.* 271:19310-19317 (1996)]. The antisera were affinity purified against m(His)₆VEGF-B₁₈₆ covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia).

20

Example 1

Procedure:

Cultures of NIH3T3 cells expressing human Flt-1 receptor protein were first starved in 0.5% fetal calf serum (FCS) in DMEM for 24 hours. The cells were then stimulated
25 for 5 minutes at 37°C in conditioned medium from cultures of 293 EBNA cells which had been transfected respectively with pREP7-hVEGF-A or pREP7-hVEGF-B₁₆₇. Conditioned medium from 293 EBNA cells transfected with pREP7 alone was used as a negative control (Mock). All conditioned media contained 1
30 µg/ml heparin. After stimulation, the cells were rinsed in ice-cold PBS containing 0.1 mM sodium orthovanadate and lysed in RIPA buffer containing 2 mM sodium orthovanadate, 1 mg/ml aprotinin and 1 mM PMSF. The lysates were sonicated, clarified by centrifugation and incubated on ice
35 for 2 hours with the anti-flt-1 antibody, SC316 (Santa Cruz). The resulting immune complexes were collected by

precipitation with protein A-sepharose beads. Immunoprecipitates were washed three times with the lysis buffer, separated by electrophoresis on 6% SDS PAGE and transferred to a nitrocellulose filter. The filter was
5 probed with horse-radish peroxide (HRP)-conjugated anti-phosphotyrosine antibody RC20H (Transduction Labs) and immunoreactivity detected by ECL (Amersham). The anti-phosphotyrosine antibody recognized phosphorylated tyrosine on Flt-1 and enabled observation of autophosphorylation of
10 the Flt-1 receptor.

Results:

As can be seen from the accompanying Figure 1, which is an anti-PTyr probed Western blot of Flt-1 immunoprecipitated
15 from Flt-1 expressing NIH3T3 cells stimulated with heparin-supplemented conditioned medium from VEGF vector, empty vector or human VEGF-B₁₆₇ pREP7 vector-transfected 293 EBNA cells, somewhat weak but nevertheless positive tyrosine phosphorylated bands are observed for both VEGF-A and VEGF-B
20 which indicate that both of these ligands cause autophosphorylation of Flt-1. In contrast, the mock (-) lane in the center of the Figure is devoid of autophosphorylation.

This data shows that human VEGF-B binds with and
25 induces autophosphorylation of the Flt-1 receptor. This indicates that Flt-1 also is a receptor for human VEGF-B₁₆₇.

Example 2

Procedure:

30 Receptor IgG fusion proteins:

cDNA encoding the first three immunoglobulin (Ig) loops of Flt-1 was spliced to the Fc region of a human IgG heavy chain and cloned in to the vector pREP7 (Invitrogen) to yield the plasmid pREP7 Flt-1-IgFc. pREP7 KDR-IgFc was
35 constructed in a similar fashion. The Flt-1-Ig Fc and KDR-Ig Fc cDNAs used in this experiment were RT-PCR products

from a plasmid construct called pBJFltKT3. The resulting plasmids were used to transfect 293 EBNA cells by the calcium phosphate method, and the resulting conditioned medium was harvested 48 hours post-transfection.

5

Receptor IgG precipitation of ^{35}S -labeled mVEGF- B_{186} :

Plasmid pSJ5 (Stratagene) encoding for murine VEGF- B_{186} (pSJ5 VEGF- B_{186}) was transfected into COS cells by electroporation, and the cells were labeled for 10 hours with ^{35}S -methionine and ^{35}S -cysteine. ^{35}S -labeled hVEGF-A16S was used as a positive control for receptor binding and was produced in 293 EBNA cells transfected with pREP7 VEGF-A and labeled as described above. About 1 ml of conditioned medium containing Flt-1-IgFc or KDR-IgFc was incubated with 40 μl of a 50% slurry of protein-A sepharose for 1 hour at 4°C under continuous agitation. Conditioned medium from mock-transfected cells was used as a negative control. The protein-A sepharose beads were collected by centrifugation, and incubated with 1 ml of conditioned medium containing ^{35}S -labeled mVEGF- B_{186} or VEGF(-A) in binding buffer (PBS, 0.5% BSA, 0.02% Tween 20, 1 $\mu\text{g}/\text{ml}$ heparin) for 3 hours at room temperature with gentle agitation. The protein-A sepharose beads were collected by centrifugation, washed twice in ice-cold binding buffer and once in 20 mM tris pH 7.5, boiled in SDS sample buffer and electrophoresed on 10% PAGE.

Results:

The results are shown in Figures 2(a) and (b), which are long and short exposures of the SDS-PAGE gels. In the Figures, lane 1 shows immunoprecipitated murine VEGF- B_{186} ; lane 2 shows Flt-1-Ig, mVEGF- B_{186} ; lane 3 shows Flt-1-Ig, mock; lane 4 shows mock, mVEGF- B_{186} ; lane 5 shows KDR-Ig, mVEGF- B_{186} ; lane 6 shows KDR-Ig, mock. To determine whether VEGF- B_{186} is a ligand for Flt-1, plasmid containing cDNA for this factor, as well as plasmid encoding VEGF(-A), or the

expression vector alone were transfected into mammalian cells, and the proteins were labeled with ^{35}S amino acids. Conditioned medium from these cells were precipitated with Flt-1-IgFc or KDR-IgFc bound to protein-A sepharose beads. A 32 kDa band [identified by the upper arrow in Fig. 2(a)] was precipitated from the VEGF-B₁₈₆ conditioned medium with Flt-1-IgFc. This band, which co-migrates with immunoprecipitated VEGF-B₁₈₆, was absent in the Flt-1-IgFc precipitation of mock transfected cells, or precipitation of VEGF-B₁₈₆ by protein-A sepharose alone. Little precipitation of this 32 kDa band was also found with KDR-IgFc.

The data clearly show the formation of complexes between the murine VEGF-B₁₈₆ and the human Flt-1 receptor.

As can also be seen from the Figs. 2(a) and (b), both Flt-1-IgFc and KDR-IgFc additionally precipitated lower molecular weight species, but these three bands also were found in conditioned medium of mock transfected cells, and are considered to represent endogenous factors produced by COS cells, possibly VEGF(-A). Of these, the band indicated by the arrow in brackets may also partially represent VEGF-B related material.

Example 3

Test of VEGF-B binding to VEGFR-1, -2 and/or -3:

To determine whether VEGF-B is a ligand for VEGFR-1 -2 or -3, 293T cells were transfected with expression plasmids for VEGF₁₆₅, mVEGF-B₁₆₇, mVEGF-B₁₈₆ or VEGF-C, and the proteins were metabolically labelled, in the presence of 50 $\mu\text{g/ml}$ heparin for VEGF₁₆₅ and VEGF-B₁₆₇, and the media was collected.

Conditioned media from all except the VEGF transfection were precleared of endogenous VEGF and VEGF/VEGF-B heterodimers and then the respective proteins were either immunoprecipitated with specific antibodies or bound to soluble receptor Ig fusion proteins containing the first five Ig-like domains of Flt-1 bound to protein A-Sepharose (PAS). The precipitated ligands were analyzed by SDS-PAGE

under reducing conditions. Approximately 50 ng of soluble receptor was used for each ligand precipitation. As shown in Fig. 3, both VEGF-B splice isoforms specifically bound to VEGFR-1 but not to VEGFR-2 or -3. Functionality of the VEGFR-2 and VEGFR-3 receptors was confirmed by the binding tests with VEGF and VEGF-C, respectively. This test shows that VEGF-B binds specifically to VEGFR-1/Flt-1, making it the third ligand identified for VEGFR-1, after VEGF and PlGF.

Two bands of 32 kD and 16 kD were precipitated from the mVEGF-B₁₈₆ conditioned medium with the specific antibody and by VEGFR-1. The 32 kD band corresponds to the glycosylated, secreted form of mVEGF-B₁₈₆ [Olofsson et al., *J. Biol. Chem.* 271:19310-19317 (1996)]. The 16 kD form is apparently a product of proteolytic processing described in further detail hereinafter.

Example 4

Further Examination of VEGF-B/VEGFR-1 Binding:

The ability of VEGF-B to bind VEGFR-1 expressed on cell surface was examined using NIH3T3-Flt1 cells. Consistent with the data obtained with soluble receptors, conditioned media from mVEGF-B₁₈₆-infected, but not mock infected High Five cells, competed for ¹²⁵I-VEGF binding to NIH3T3-Flt1 as seen in Fig. 4. Half maximum inhibitory concentration for mVEGF-B₁₈₆ was estimated using quantitative immunoblots to 3 ng/ml compared to recombinant mVEGF₁₆₄ which competed for iodinated hVEGF₁₆₅ at a half maximum inhibitory concentration at 1.5 ng/ml (Figs. 4 and 5). This effect was specific to VEGFR-1 as no competition was observed with PAE-KDR cells. Thus it is apparent that although VEGF-B₁₈₆, like VEGF₁₂₁, lacks the C-terminal basic residues found in VEGF₁₆₅, it nevertheless binds to VEGFR-1 on NIH3T3-Flt1 cells. VEGF-B also was found to bind equally well to the VEGFR-1 Ig-fusion containing the three N-terminal Ig-like domains of VEGFR-1 (residues 1-347) as well as to the VEGFR-1 Ig-fusion containing five of the Ig-like domains.

Due to aggregation and protein stability problems, purified His-tagged VEGF-B (human and mouse (His)₆VEGF-B₁₈₆, also C-terminal deletion mutants covering exons 1-5 of mouse and human (His)₆VEGF-B) competed only at high concentrations with iodinated VEGF for VEGFR-1 binding.

Example 5

VEGF Competition Studies:

mVEGF-B₁₆₇ and mVEGF-B₁₈₆ expressed in transfected 293-T cells were labelled and precleared as described in the binding test, with the only difference being that 10 µg/ml heparin was used for mVEGF-B₁₆₇ in the labelling media. 2 µg of recombinant hVEGF₁₆₅ was added as a cold competitor to the binding reaction when indicated. Equal volumes of metabolically labelled factors were bound to soluble VEGFR-1 or immunoprecipitated with affinity purified N-terminal peptide VEGF-B antibody for 2 hours and washed twice with ice-cold 10mM Tris-HCl pH 8.0, 1% TritonX-100, 25 mM EDTA, 1mM PMSF and twice with PBS containing 1mM PMSF. The precipitates were analyzed by 15% SDS-PAGE. As shown in Fig. 6, the binding of these two forms as well as that of mVEGF-B₁₆₇ to VEGFR-1 was abolished by excess rhVEGF, thereby indicating that binding of VEGF-B to VEGFR-1 can be competed by excess VEGF. This test result confirms the specificity of the interaction and suggests that the interaction sites for VEGF and VEGF-B on the receptor must be overlapping, partially overlapping or at least in close proximity.

Example 6

Competition for Binding to Cell Surface Receptors:

For the competition assays, High five cells were infected with the recombinant virus for native mVEGF-B₁₈₆ (mVEGF-B₁₈₆ pFASTBAC1) and with a mock virus, and the media were harvested 48 hours post infection and immediately used or frozen at -70°C.

Recombinant mVEGF₁₆₄ (obtained from Dr. Herbert Weich) or hVEGF₁₆₅ (R&D systems or Peprotech) were labelled with ¹²⁵I using the Iodo-Gen reagent (Pierce) and purified by gel filtration on PD-10 columns (Pharmacia). The specific activities were 2.2x10⁵ cpm/ng and 1.0x10⁵ cpm/ng for mVEGF and hVEGF, respectively. For binding analysis PAE-KDR and NIH3T3-Flt1 cells were seeded in 24 well plates coated with 0.2% gelatin, grown to confluence, washed twice with ice-cold binding buffer (Ham's F12, 0.5mg/ml BSA, 10 mM Hepes pH 7.4 for PAE-KDR and DMEM, 0.5 mg/ml BSA, 10 mM Hepes pH 7.4 for NIH3T3 Flt1) and incubated in triplicate with 0.5 ng/ml [¹²⁵I]-VEGF in binding buffer containing increasing amounts of unlabelled VEGF or media from VEGF-B or mock infected insect cells. After incubation for 2 hours at + 4°C, the cells were washed three times with ice-cold binding buffer and twice with PBS containing 0.5 mg/ml BSA and lysed in 0.5 M NaOH. The solubilized radioactivity was measured using a gamma counter. Fig. 4 shows displacement of [¹²⁵I]-hVEGF₁₆₅ from VEGFR-1/Flt1 by mVEGF-B₁₈₆ using NIH 3T3 Flt-1 cells. Fig. 5 shows competition on NIH-VEGFR-1/Flt-1 by mVEGF₁₆₄.

In an analogous test, mVEGF-B₁₈₆ was found not to compete with [¹²⁵I]-VEGF for VEGFR-2 on PAE-KDR cells.

Competition analysis using purified recombinant (His)₆VEGF-B₁₈₆ indicated that only a minor portion of the protein is biologically active, since the native (own signal sequence) unpurified VEGF-B₁₈₆ competed far more efficiently with iodinated VEGF for VEGFR-1 binding.

Example 7

Proteolytic Processing of VEGF-B₁₈₆:

mVEGF-B₁₈₆ expressed in COS cells is modified by O-linked glycosylation, which increases the apparent molecular weight from 25 kDa of the intracellular form to 32 kDa in the secreted form. As noted above, when mVEGFB₁₈₆ was expressed in 293-T cells, a faster form migrating as a 16 kDa band

appeared in addition to the 32 kDa form. This band was also observed in conditioned media from COS cells when the cells were labeled for a longer period. The following test was carried out to compare the migration of dimers formed by mVEGF-B₁₈₆ to mVEGF-B₁₆₇, and a C-terminal truncated form mVEGF-B_{KEx1-5} expressed in 293-T cells and their ability to bind the sVEGFR-1. The mVEGF-B exon 1-5 mutant containing a C-terminal Kemptide motif [Mohanraj et al., *Protein Expression & Purification*, 8:175-82 (1996)] (mVEGF-B_{KEx1-5} pSG5, was produced by polymerase chain reaction (PCR).

VEGF-B_{KEx1-5}, VEGF-B₁₆₇ and VEGF-B₁₈₆ were expressed in 293-T cells. The cells were metabolically labelled. Mock transfected and VEGF-B₁₆₇ transfected cells were labelled in the presence of 10 µg/ml heparin. The collected media were precleared of VEGF and heterodimers, and were immunoprecipitated with an affinity purified N-terminal VEGF-B peptide antibody or bound to VEGFR-1. The bound ligands were analyzed by SDS-PAGE under non-reducing conditions. The results are shown in Fig. 7.

It can be seen that mVEGF-B₁₈₆ migrates as three different dimeric polypeptides the shortest being 34 kDa, an intermediate form of 48 kDa and the full length form of 60kDa. The 34 kDa band migrates slightly slower than mVEGF-B_{KEx1-5}, indicating that the putative cleavage site is more C-terminal, presumably in the beginning of the translated exon 6A [Olofsson et al., *J. Biol. Chem.* 271:19310-19317 (1996)].

The test clearly shows that the longer VEGF-B₁₈₆ isoform undergoes proteolytic processing which results in a shorter form containing the receptor binding epitopes for VEGFR-1. The functional aspects of this proteolytic processing of VEGF-B₁₈₆ are not fully understood. Since the VEGF-B₁₈₆ isoform is readily secreted from cells, the proteolytic processing does not appear to be a way of regulating the release or availability of the protein.

As can be seen from Fig. 7, the relative intensity of the VEGF-B signal compared between the immunoprecipitated forms and the receptor-bound forms shows that the strength of the signal seems to correlate with the level of processed subunits in the dimers, thereby indicating that processing leads to an increased affinity for the receptor. The 48 kDa band is believed to consist of a dimer between a processed (16 kDa) and a full length (32 kDa) monomer. The 34 kDa band consists of a dimer between two processed monomers of 16 kDa each. It is significant that both these dimers which comprise the 16 kDa analog produced by processing of VEGF-B, bind better to the VEGFR-1 receptor than the ~60 kDa dimer which is made up of two full length 32 kDa monomers.

Example 8

Plasmin Cleavage:

The following test was run to determine whether a full length form of VEGF-B₁₈₆ expressed in COS cells could be cleaved by the addition of plasmin, and if this affects the VEGFR-1 binding. This could be a physiological mechanism at the site of basement membrane degradation in the angiogenesis process.

COS-7 cells were transfected with mVEGF-B₁₈₆, metabolically labelled for 75 minutes, and the collected media was precleared of VEGF and heterodimers. The media was then incubated at 37°C with 0.1 U/ml plasmin (Boehringer Mannheim) for the time periods of 0, 5, 15, 30 and 60 minutes. The reaction was stopped by addition of 1 mM PMSF and 0.1 casein units of aprotinin. The media were immunoprecipitated by the affinity purified N-peptide VEGF-B antibody and also bound to VEGFR-1 Ig. The precipitated proteins were analyzed by SDS-PAGE under reducing conditions. The results are shown in Fig. 8.

Concomitant with the reduced amounts of the full length form is the appearance a 15 kDa fragment followed by a secondary fragment of 12 kDa. Thus plasmin cleavage does

occur, but evidently does not give rise to the same fragment as the endogenous proteolytic processing of VEGF-B₁₆₆ described above. Nevertheless, this N-terminal fragment is fully capable of interacting with sVEGFR-1, suggesting that VEGF-B is similar to VEGF, in that the receptor binding epitopes are contained in the N-terminal fragment which is resistant to proteases such as plasmin.

Example 9

10 Mutational Analysis of Receptor Epitopes:

Crystal structure determination and mapping of the VEGFR-2 epitope for VEGF has pointed to a number of hot spot amino acid residues, with the most important residues for the ligand-receptor interaction being Ile-46, Ile-83, Glu-64, Phe-17, Gln-79, Pro-85, Ile-43 and Lys-84 [Muller et al., *Proc. Natl. Acad. Sci. USA* 94:7192-7 (1997)]. The extent to which these residues are involved in VEGFR-1 binding is less clear. By charged amino acid to alanine scan mutagenesis [Keyt et al., *J. Biol. Chem.*, 271:5638-5646 (1996)] the VEGFR-1 binding epitope in VEGF was proposed to involve a stretch of acidic residues (Asp-63, Glu-64 and Glu-67). These amino acid residues are conserved in VEGF-B (Asp-63, Asp-64 and Glu-67) and to a lesser extent in PlGF.

In order to analyze whether the acidic amino acid residues which are conserved between VEGF and VEGF-B and which have been implicated in VEGF/VEGFR-1 binding, are also are the major determinants for VEGF-B/VEGFR-1 binding, Asp63 Asp64 and Glu67 were mutated into alanines. The mutation scheme is illustrated in Fig. 9, which is a schematic illustration of the wildtype VEGF-B forms and the different mutants.

The putative receptor epitope mutants of VEGF-B₁₆₇ were expressed in transfected 293-T cells and metabolically labelled in the presence of 50 µg/ml heparin. In order to study the VEGF-B homodimers, endogenous VEGF and VEGF heterodimers formed by VEGF and overexpressed VEGF-B were

immunodepleted with VEGF antibodies (MAB 293 from R&D Systems). The VEGF-B mutants were either immunoprecipitated with affinity purified N-terminal peptide antibody or bound to soluble VEGFR-1 Ig. The precipitates were analyzed by SDS-PAGE under reducing conditions. From the results it is apparent that neither mutation of two first acidic residues nor the mutation of all three acidic amino acid residues abolished VEGF-B binding to VEGFR-1. Thus, this data based upon either mutation of all three charged amino acids to alanines or on mutation of only the first two charged amino acid residues into alanines, indicates that the conserved acidic residues are not the major contributors to the binding of VEGF-B to VEGFR-1.

Example 10

Mutational Analysis of Conserved Cysteines in VEGF-B₁₆₇:

To examine the contribution of the conserved cysteines to dimer formation of VEGF-B and test the structural prediction based upon the anti-parallel covalent VEGF dimer model, cysteine-51 (Cys 2) and cysteine-60 (Cys 4) were mutated to serine residues. The cysteine to serine mutants in mVEGF-B₁₆₇, pSG5 were generated by M13-based in vitro single stranded mutagenesis employing the helper phage M13K07 [Viera et al., *Methods Enzymol.*, 153:3-11 (1987)] and the *dut⁻ ung⁻* *E.coli* strain RZ1032 [Kunkel et al., *Methods Enzymol.* 154:367-382 (1987)]. Mutations were carried out both as single mutants (C2S and C4S) and as a double mutant (C2S,C4S). The mutation scheme is illustrated in Fig. 9.

The mutants and wildtype VEGF-B₁₆₇ were expressed alone or in different combinations as co-transfections and metabolically labelled in 293-T cells, and the media were precleared from VEGF and heterodimers. The results are shown in Figs. 10a-c. The media were either immunoprecipitated with the affinity purified N-terminal VEGF-B antibody and analyzed under both non-reducing conditions (Fig. 10a) and reducing conditions (Fig. 10b) or

bound to soluble VEGFR-1 Ig (Fig. 10c). As can be seen from Fig. 10b, all the mutants were expressed in approximately same amounts.

5 It was found that VEGF-B₁₈₆ is cleaved, most likely C-terminal of the region identical in the two splice variants, which is encoded by exons 1-5 and contains the cysteine knot as well as the receptor binding epitopes. Wildtype VEGF-B₁₆₇ migrated under non-reducing conditions as two bands 42 kDa and 46 kDa, however only the 46 kDa form
10 was capable of binding to the VEGFR-1 (compare Figs. 2A and 3C). The 42 kD band is believed to correspond to dimers joined together by aberrant disulfide bridges, since these doublet bands are not seen with VEGF-B₁₈₆ or VEGF-B_{kEx1-5}, which lack the additional eight cysteines found in the C-terminal
15 part of VEGF-B₁₆₇. The single mutant C4S gave rise to monomers. Also some dimers migrating as a 42kDa band were observed which were unable to bind to VEGFR-1. Surprisingly the C2S mutant, although partly produced as monomers, could still form dimers capable of receptor binding. Co-
20 transfection of the single mutants (C2S+C4S) led to increased amounts of the 46kDa band regaining receptor binding, indicating that the dimerization impairment can be complemented by establishing a disulfide link between the non-mutated cysteins similar to VEGF [Potgens et al., *J. Biol. Chem.*, 269:32879-85 (1994)]. Co-transfection of a
25 single mutant with the double mutant failed to complement.

Some of the VEGF-B₁₆₇ mutants were expressed as above, and cells were labelled in the presence of 10 µg/ml heparin. The collected media were incubated with VEGFR-1 Ig, and the
30 bound ligands were subjected to SDS-PAGE under non-reducing conditions. The results are shown in Fig. 11. It can be seen that the C4S mutant and the double mutant C2SC4S showed residual receptor binding which is explainable by the interactions of the soluble receptor to the monomers.

Thus, the mutational analysis of conserved cysteines which contribute to the formation of VEGF-B dimers indicates a structural conservation with VEGF and PDGF.

5

Example 11

Biological Response to VEGF-B:

RT-PCR analysis using specific primers based on the bovine VEGFR-1 sequence shows that VEGFR-1 mRNA is expressed by bovine adrenal cortex-derived microvascular endothelial (BME) and bovine aortic endothelial (BAE) cells. To determine the biological response of VEGF-B on endothelial cells, replicate filters containing 5 µg/lane of total cellular RNA prepared from confluent monolayers of BME cells incubated in the presence of 50 ng/ml hVEGF-B₁₈₆ were hybridized with [³²P]-labelled cRNA probes. BME cells [Furie et al., *J. Cell Biol.*, 98:1033-41 (1984)] were grown in MEM alpha modification (Gibco AG, Basel, Switzerland) supplemented with 15% donor calf serum on 1.5% gelatin coated tissue culture flasks. The cytokine was added to confluent monolayers of BME cells to which fresh complete medium had been added 24 hours previously. Total cellular RNA was prepared after time periods of 0, 1, 3, 9, 24 and 48 hours using Trizol reagent (Life Technologies AG, Basel, Switzerland). Northern blots, UV-cross linking and methylene blue staining of filters, in vitro transcription, hybridization and post hybridization washes were carried out as described in [Pepper et al., *J. Cell Biol.*, 111:743-55 (1990)]. The ³²P-labelled cRNA probes were prepared from bovine u-PA [Kratzschmar et al., *Gene*, 125:177-83 (1993)], human t-PA [Fisher et al., *J. Biol. Chem.*, 260:11223-30 (1985)] and bovine PAI-1 [Pepper et al., *J. Cell Biol.*, 111:743-55 (1990)] cDNAs as described in [Pepper et al., *J. Cell Biol.*, 111:743-55 (1990); Pepper et al., *J. Cell Biol.*, 122:673-84 (1993)]. The results are shown in Fig. 12. RNA integrity and uniformity of loading were determined by staining the filters with methylene blue after transfer and

cross-linking (lower panel of the figure); 28S and 18S ribosomal RNAs are shown.

The Northern blot analysis showed that VEGF-B₁₈₆ (50 ng/ml) increased steady state levels of urokinase type plasminogen activator (u-PA) and plasminogen activator inhibitor 1 (PAI-1) mRNAs in BME cells. That is, the test showed that endothelial cells responded to VEGF-B by inducing PAI-1 mRNA and u-PA mRNA. Thus, binding of VEGF-B to its receptor on endothelial cells stimulates the activity of u-PA as well as of PAI-1, which are important modulators of extracellular matrix degradation and cell adhesion and migration.

Example 12

15 Zymography and Reverse Zymography:

Cell extracts prepared from BME cells incubated in the presence of hVEGF-B₁₈₆ at concentrations of 0, 1, 3, 10, 30 and 100 ng/ml, VEGF at a concentration of 30 ng/ml, or recombinant human bFGF (155 amino acid form obtained from Dr. P. Sarmientos) at a concentration of 30 ng/ml, were subjected to zymography and reverse zymography as follows. Confluent monolayers of BME cells in 35 mm gelatin coated tissue culture dishes were washed twice with serum free medium and the cytokines were added in serum free medium containing trasylol (200 KIU/ml). After 15 hours incubation time, cell extracts were prepared and analysed by zymography and reverse zymography as described in Vassalli et al., *J. Exp. Med.*, 159:1653-68 (1984) and in Pepper et al., *J. Cell Biol.*, 111:743-55 (1990). The results are shown in Figs. 13a-b and indicate that recombinant hVEGF-B₁₈₆ increases u-PA and PAI-1 activity in BME cells. The Fig. 13a shows a zymographic analysis and Fig. 13b shows a reverse zymography analysis of cell extracts from BME cells. It can be seen that VEGF-B₁₈₆ induces a dose-dependent increase in u-PA and PAI-1 activity in the BME cells. The apparent lack of induction of PAI-1 activity by VEGF used as a control,

reflects rapid sequestration of PAI-1 into a complex with VEGF-induced tPA. This complex is observed by zymography of the culture supernatant of VEGF-treated cells. In contrast to VEGF [Pepper et al., *Biophys. Res. Commun.*, 189:824-31 (1992)], VEGF-B₁₈₆ did not increase t-PA activity. The test showed that endothelial cells responded to VEGF-B by increasing synthesis of u-PA and PAI-1 and the resultant protein activities. However, the kinetics of PAI-1 induction were more rapid (within 1 hour) and transient (maximal effect observed at 3 hours) than those of u-PA (induced after 9 hours and sustained for up to 48 hours). This is in agreement with what has been observed for bFGF and VEGF [Pepper et al., *J. Cell Biol.*, 111:743-55 (1990); Pepper et al., *Biochem. Biophys. Res. Commun.*, 181, 902-906 (1991); Mandriota et al., *J. Biol. Chem.*, 270:9709-16 (1995)].

Examples 11 and 12 show that recombinant hVEGF-B₁₈₆ increases steady-state levels of u-PA and PAI-1 mRNAs in BME cells. In similar testing, VEGF-B₁₈₆ also induced PAI-1 but not u-PA mRNA in BAE cells.

Usefulness

The formation of complexes between Flt-1 tyrosine kinase receptors and VEGF-B and/or VEGF-B analogs may be used as a treatment for disease states characterized by overexpression of the Flt-1 receptor by administering to a patient suffering from such a disease state an effective Flt-1 receptor binding or receptor antagonizing amount of VEGF-B or a VEGF-B analog. An example of such a disease state characterized by overexpression of the Flt-1 receptor is hemangioendothelioma. The Flt-1 receptor also is overexpressed in various tumors [Warren et al., *J. Clin. Invest.*, 95(4):1789-97 (1995); Hatva et al., *Amer. J. Pathology*, 146(2):368-78 (1995)]. The formation of complexes between VEGFR-1 and VEGF-B or a VEGF-B analog may also be useful in treating states characterized by

underexpression on a Flt-1 receptor. Such states may include normal adult endothelium or states which require increased blood vessel formation. The amount to be administered in a given case will depend on the characteristics of the patient and the nature of the disease state and can be determined by a person skilled in the art by routine experimentation.

The VEGF-B or VEGF-B analog may suitably be administered intravenously or by means of a targeted delivery system analogous to the systems heretofore used for targeted delivery of VEGF or FGF. Examples of such systems include use of DNA in the form of a plasmid [Isner et al., *Lancet*, 348:370 (1996)] or use of a recombinant adenovirus [Giordano et al., *Nature Medicine*, 2:534-39 (1996)]. VEGF-B could also be provided in protein form by techniques analogous to those described for VEGF [Bauters et al., *The American Physiological Society*, pp H1263-271 (1994); Asahara et al., *Circulation*, 91:2793 (1995)] or through use of a defective herpes virus [Mesri et al., *Circulation Research*, 76:161 (1995)]. Small molecule VEGF-B analogues could be administered orally. Other standard delivery modes, such as sub-cutaneous or intra-peritoneal injection, could also be used.

VEGF-B protein/Flt-1 receptor complexes also can be used to produce antibodies. The antibodies may be either polyclonal antibodies or monoclonal antibodies. In general, conventional antibody production techniques may be used to produce antibodies to VEGF-B/Flt-1 complexes. For example, specific monoclonal antibodies may be produced via immunization of fusion proteins obtained by recombinant DNA expression. Both chimeric and humanized antibodies and antibody fragments to the VEGF-B/Receptor complex are expressly contemplated to be within the scope of the invention. Labelled monoclonal antibodies, in particular, should be useful in screening for medical conditions characterized by overexpression or underexpression of the

Flt-1 receptor. Examples of such conditions include endothelial cell tumors of blood and lymphatic vessels, for example, hemangioendothelioma.

5 In one preferred embodiment of a diagnostic/prognostic means according to the invention, either the antibody, the growth factor or the receptor is labelled, and one of the three is substrate-bound, such that the antibody-complex interaction can be established by determining the amount of label attached to the substrate following binding between
10 the antibody and the growth factor/receptor complex. In a particularly preferred embodiment of the invention, the diagnostic/prognostic means may be provided as a conventional ELISA kit.

The foregoing description and examples have been set
15 forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the described embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all
20 variations falling within the scope of the appended claims and equivalents thereof.

The following references provide technical background information and are hereby incorporated herein by reference:

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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 OLOFSSON, Birgitta
 GUNJI, Yuji
 ERIKSSON, Ulf
 ALITALO, Kari
- (ii) TITLE OF INVENTION: VEGF-B/RECEPTOR COMPLEX AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
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 - (C) CITY: Washington
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 - (F) ZIP: 20005
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: WO
 - (B) FILING DATE: 19-DEC-1997
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/033,697
 - (B) FILING DATE: 20-DEC-1996
- (viii) ATTORNEY/AGENT INFORMATION:
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- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202) 628-8800
 - (B) TELEFAX: (202) 628-8844

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGTACCCC CAGGCTCAGC ATACAAAAAG AC

32

(2) INFORMATION FOR SEQ ID NO:2:

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 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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60

TT

62

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 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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CAAGGGCGGG GCTTAGAGAT CTAGCT

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 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAATTCCCC GCCCAGGCCC CTGTC

25

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 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAATTCAAT GATGATGATG ATGATGAGCC CCGCCCTTGG C

41

What is claimed is:

1. A method for identifying a VEGF-B analog, said analog being characterized as having substantially the same binding affinity for a cell surface receptor as VEGF-B, said method comprising the steps of:

- 5 (a) providing a sample containing a receptor protein selected from the group consisting of:
- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
 - 10 (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; and
 - 15 (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;
- (b) contacting said sample of step (a) with a
20 candidate VEGF-B analog; and
- (c) detecting specific binding between said candidate VEGF-B analog and the receptor protein of step (a).

2. A method for identifying a VEGF-B analog, said analog being characterized as having substantially the same binding affinity for a cell surface receptor as VEGF-B, said method comprising the steps of:

- 5 (a) providing a sample containing cells that express a surface receptor protein having binding affinity for VEGF-B selected from the group consisting of:
- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
 - 10

- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; and
- 15 (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;
- 20 (b) contacting said cells with a candidate VEGF-B analog, and
- (c) detecting induction of a VEGF-B-mediated cellular response.

3. A method according to claim 2, wherein said VEGF-B-mediated cellular response detected in step (c) is endothelial cell proliferation.

4. A method according to claim 2, wherein said VEGF-B-mediated cellular response is angiogenesis.

5. A kit for identifying VEGF-B or a candidate VEGF-B analog in a sample, said kit comprising:

- (a) a receptacle adapted to receive a sample and containing a receptor protein selected from the group
- 5 consisting of:
 - (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
 - (ii) a polypeptide chain having binding affinity
 - 10 for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; and
 - (iii) a polypeptide chain having binding affinity
 - 15 for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a

nucleic acid comprising the sequence defined
by nucleotides 1-1293 of Flt-1;

and

20 (b) means for detecting interaction of VEGF-B or a
candidate VEGF-B analog with said receptor protein contained
in said receptacle, said VEGF-B or candidate VEGF-B analog
comprising part of a sample received in said receptacle.

6. A kit according to claim 5, wherein said detecting
means comprises means for detecting specific binding
interaction of VEGF-B or a VEGF-B analog with said receptor
protein.

7. A kit according to claim 5, wherein said detecting
means comprises means for detecting induction of a VEGF-B-
mediated cellular response.

8. An isolated ligand-receptor complex comprising two
molecules, one of said molecules defining said ligand and
comprising at least amino acids 1-115 of VEGF-B or a
receptor-binding analog thereof, and a second of said
5 molecules defining said receptor and being selected from the
group consisting of:

- (i) a polypeptide chain comprising an amino acid
sequence defined by residues 1-347 of Flt-1, or a
VEGF-B-specific receptor analog thereof;
- 10 (ii) a polypeptide chain having binding affinity for
VEGF-B and sharing at least 30% amino acid
identity with residues 1-347 of Flt-1;
- (iii) a polypeptide chain having binding affinity for
15 VEGF-B and encoded by a nucleic acid that
hybridizes under stringent conditions with a
nucleic acid comprising the sequence defined by
nucleotides 1-1293 of Flt-1.

9. A complex according to claim 8, wherein said receptor also has binding affinity for VEGF.

10. A complex according to claim 8, wherein said ligand is VEGF-B or a VEGF-B analog having at least 50% amino acid sequence identity to amino acids 1-115 of VEGF-B or a receptor-binding amino acid sequence variant or xenogeneic homolog thereof.

11. An isolated binding partner having specific binding affinity for an epitope on a ligand-receptor complex, said complex comprising VEGF-B protein or an analog thereof in specific binding interaction with the ligand binding domain of a cell surface receptor defined by:

- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; or
- (iii) a polypeptide chain having binding affinity for VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;

said binding partner having substantially no binding affinity for uncomplexed VEGF-B or VEGF-B analog.

12. An isolated binding partner according to claim 11, wherein said binding partner has substantially no binding affinity for an uncomplexed form of said cell surface receptor protein or a receptor analog thereof.

13. A binding partner according to claim 11, wherein said binding partner is an antibody.

14. A binding partner according to claim 13, wherein said binding partner is a monoclonal antibody.

15. A binding partner according to claim 13, wherein said binding partner is a polyclonal antibody.

16. Use of a VEGF-B analog obtained according to the method of claim 1 or claim 2 in a method for

- (i) antagonizing VEGF-B binding to a cell surface receptor, or
- 5 (ii) antagonizing induction of a VEGF-B-mediated cellular response.

17. The use according to claim 16 wherein said VEGF-B analog comprises an antibody having binding specificity for

- (i) the ligand binding domain of a cell surface receptor defined by amino acids 1-347 of Flt-1 or
- 5 a VEGF-B-specific receptor analog thereof, or
- (ii) a receptor binding domain of VEGF-B, represented by amino acids 1-115 of VEGF-B, or a receptor-binding analog thereof.

18. Use of a receptor protein selected from the group consisting of:

- (i) a polypeptide chain comprising an amino acid sequence defined by residues 1-347 of Flt-1, or a
- 5 VEGF-B-specific receptor analog thereof;
- (ii) a polypeptide chain having binding affinity for VEGF-B and sharing at least 30% amino acid identity with residues 1-347 of Flt-1; or
- (iii) a polypeptide chain having binding affinity for
- 10 VEGF-B and encoded by a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising the sequence defined by nucleotides 1-1293 of Flt-1;

in a method for antagonizing:

- 15 (a) VEGF-B binding to a cell surface receptor, or
(b) induction of a VEGF-B-mediated cellular response.

19. A method for antagonizing VEGF-B binding to a cell surface receptor, said method comprising the step of providing a protein having binding specificity for the amino acid sequence defined by residues 1-347 of Flt-1 or a VEGF-B
5 receptor binding sequence variant thereof, said protein having at least 50% amino acid sequence identity with residues 1-115 of VEGF-B, such that said protein, when provided to a cell expressing said cell surface receptor, is competent to interact specifically with said receptor,
10 thereby substantially inhibiting VEGF-B binding to said receptor.

20. A method according to claim 18, wherein said protein is a VEGF-B analog obtained according to the method of claim 1 or claim 2.

21. A method for treating a disease state characterized by overexpression of an Flt-1 cell surface receptor, said method comprising administering to a patient suffering from said disease state an effective receptor-
5 binding amount of a VEGF-B antagonist, wherein said VEGF-B antagonist comprises a VEGF-B analog obtained according to the method of claim 1 or claim 2, or an antibody to VEGF-B.

22. A method for treating a state characterized by
10 underexpression of an Flt-1 cell surface receptor, said method comprising administering to a patient in said state an effective receptor binding amount of VEGF-B or a VEGF-B agonist, said VEGF-B agonist comprising a VEGF-B analog obtained according to the method of claim 1 or claim 2.

15

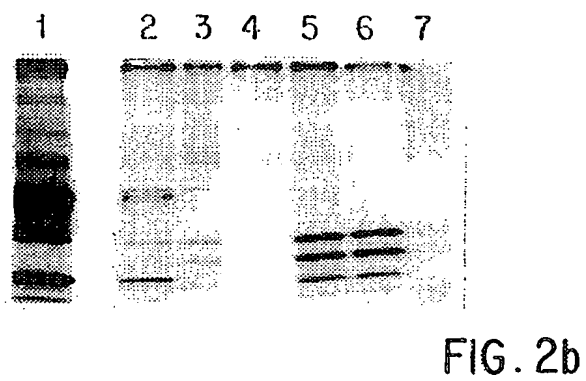
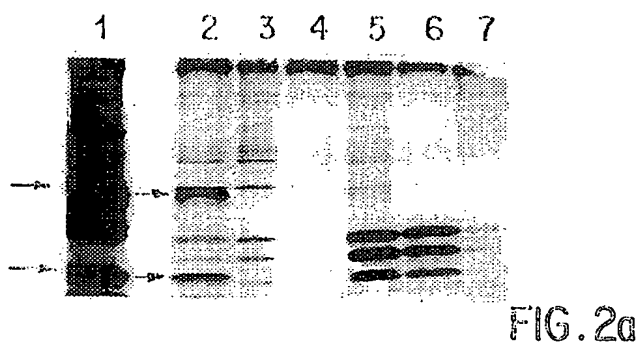
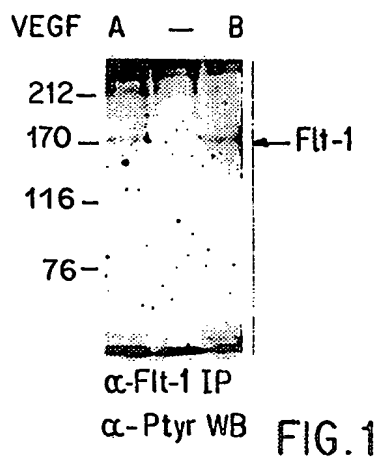
23. A VEGF-B analog which comprises a VEGFR-1 binding fragment of VEGF-B.

24. A VEGF-B analog according to claim 23, wherein
said analog is selected from the group consisting of a
20 receptor-binding 16 kDa fragment produced by proteolytic
processing of VEGF-B, a receptor-binding fragment produced
by plasmin digestion of VEGF-B, a receptor-binding exon 1-5
mutant fragment containing a C-terminal Kemptide motif, and
25 dimers comprising at least one of said receptor-binding
fragments.

25. A VEGF-B analog according to claim 23, which
comprises a dimer of two 16 kDa receptor-binding fragments
obtained by proteolytic processing of VEGF-B.

26. A VEGF-B analog according to claim 23, which is a
dimer of a full-length VEGF-B monomer and a 16 kDa receptor-
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VEGF-B.

27. A polynucleotide encoding a VEGF-B analog
according to claim 24.



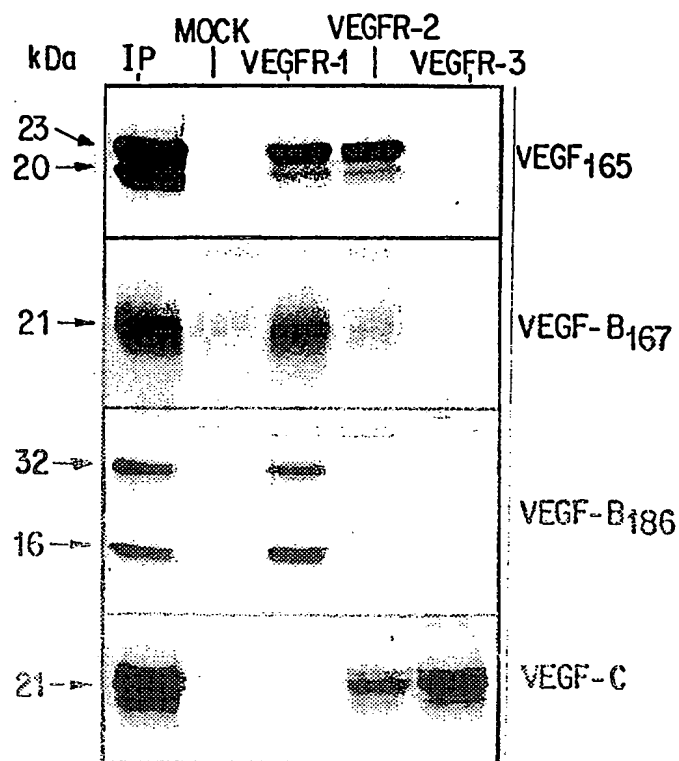


FIG. 3

FIGURE 4

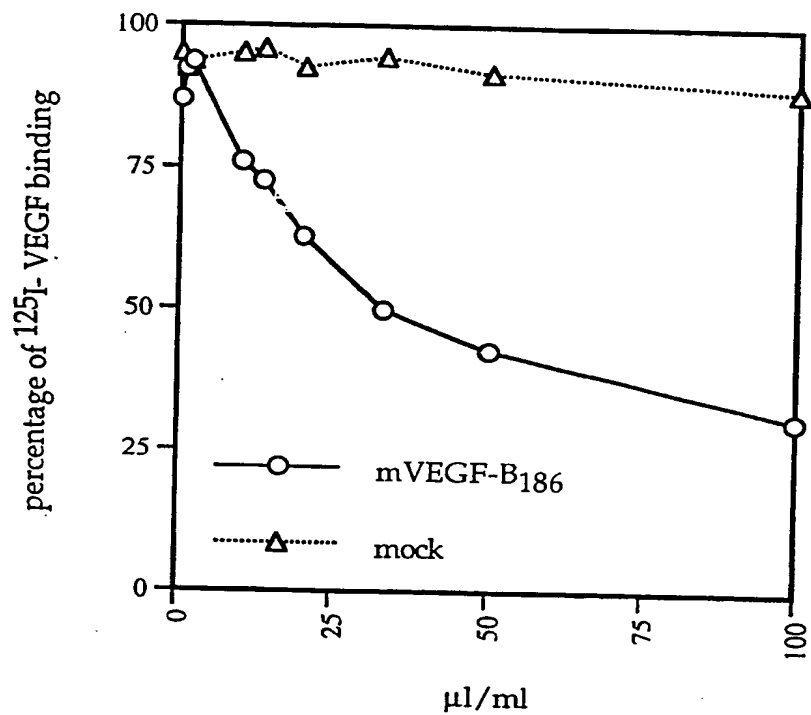
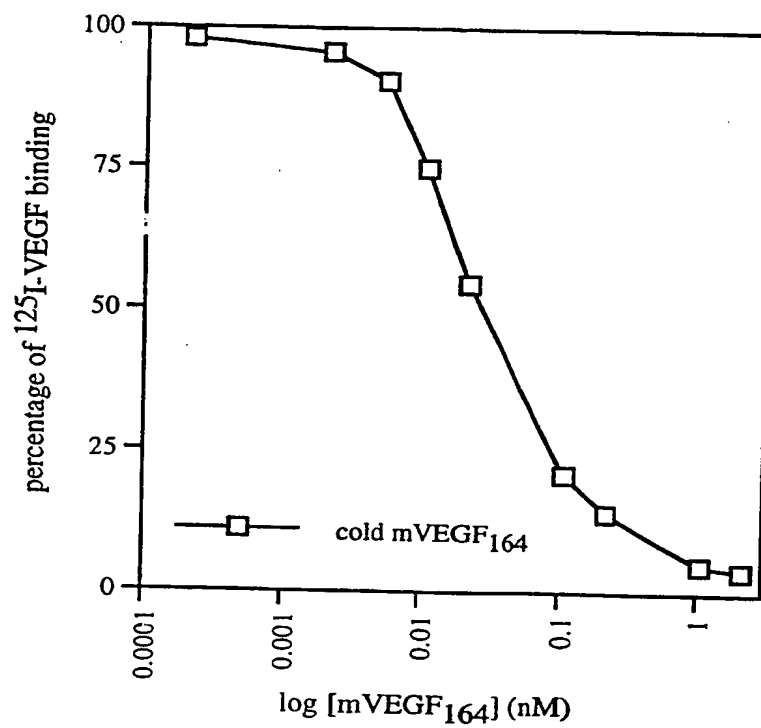


FIGURE 5



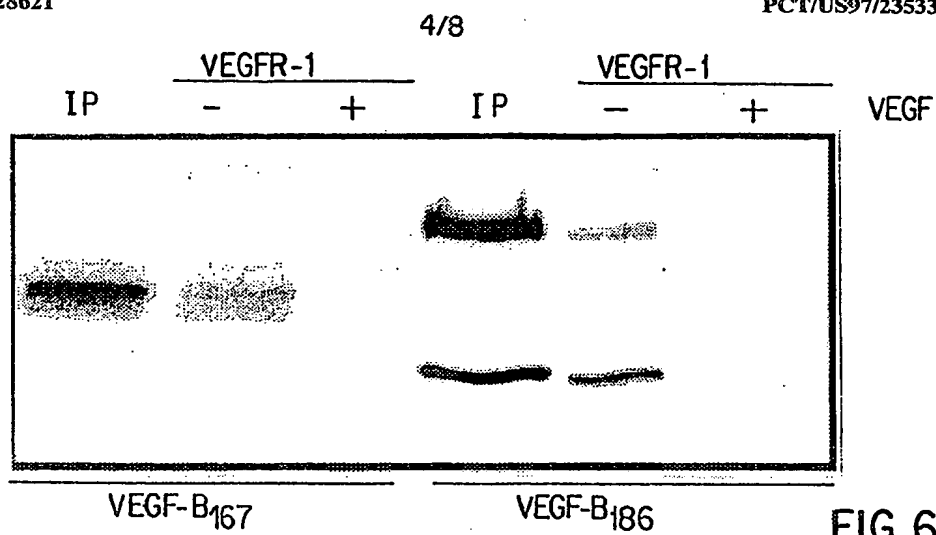


FIG. 6

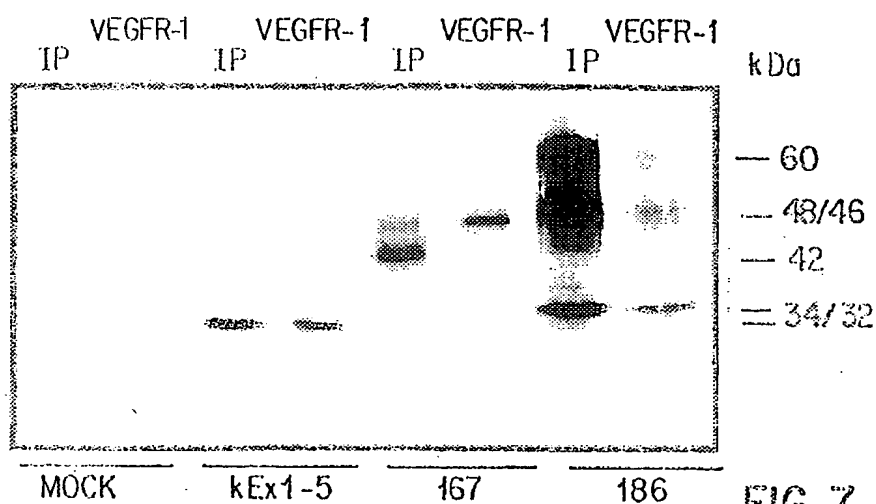


FIG. 7

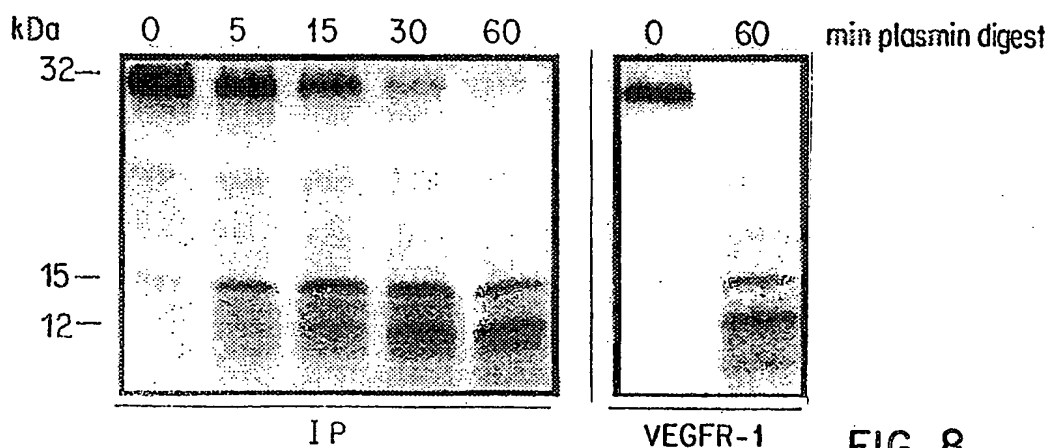
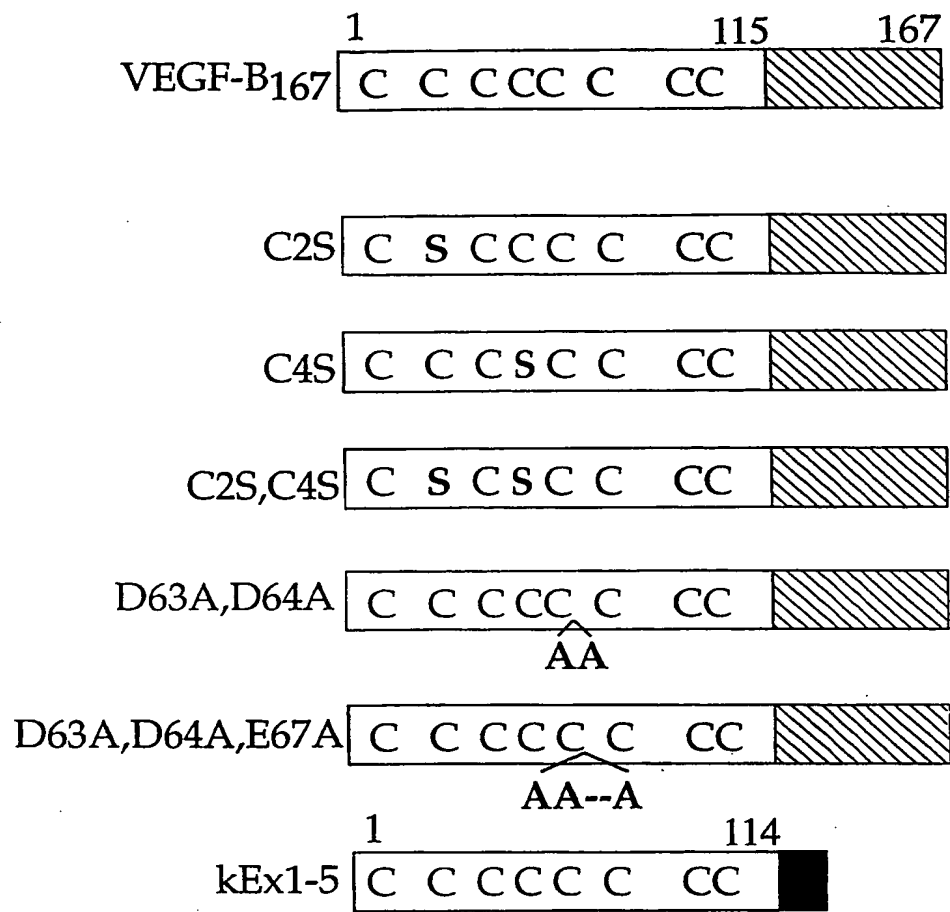


FIG. 8

FIGURE 9



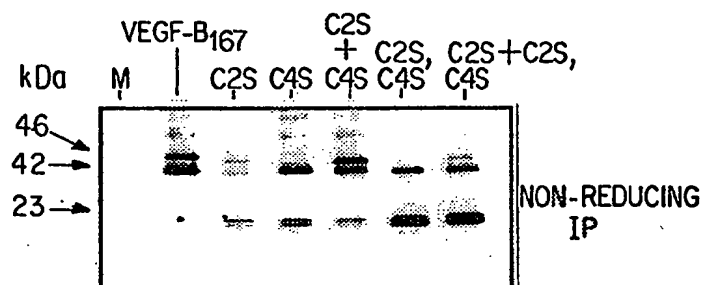


FIG. 10a

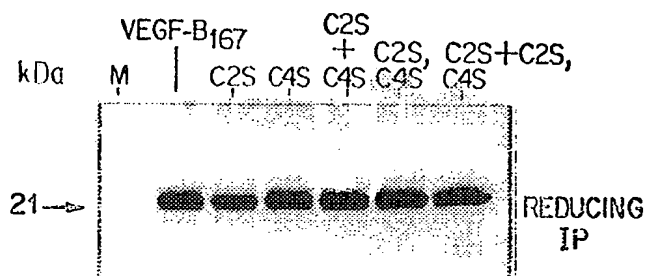


FIG. 10b

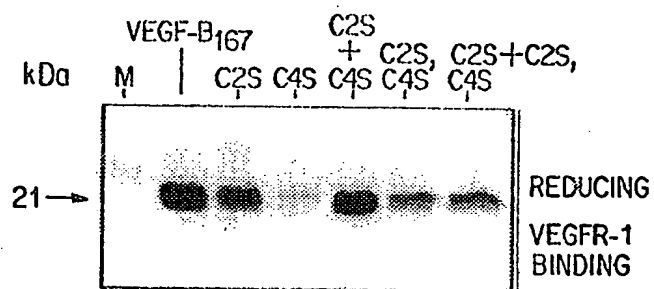


FIG. 10c

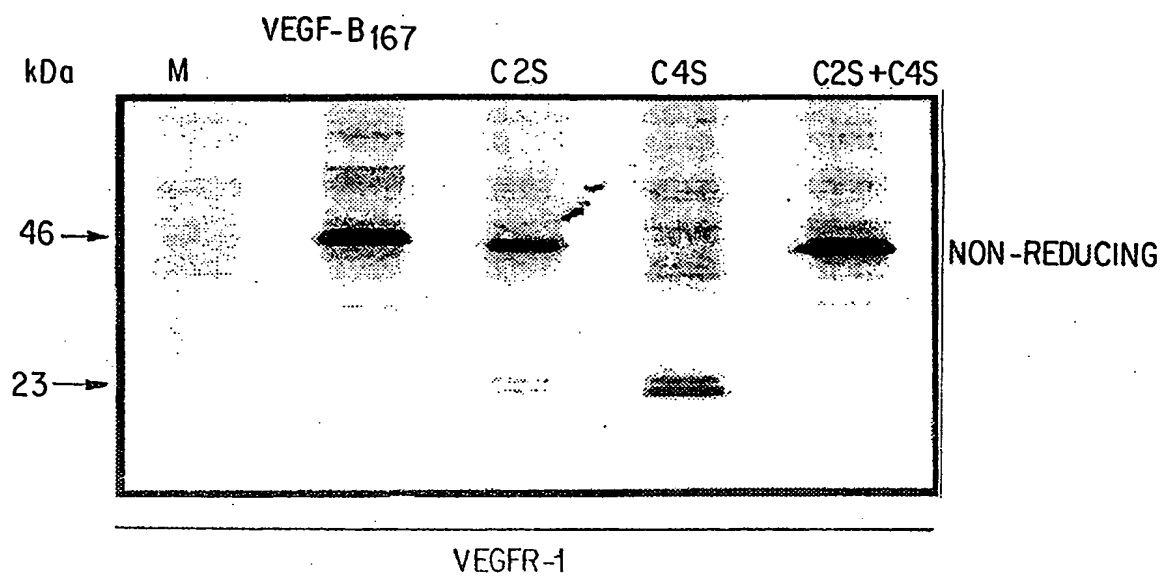
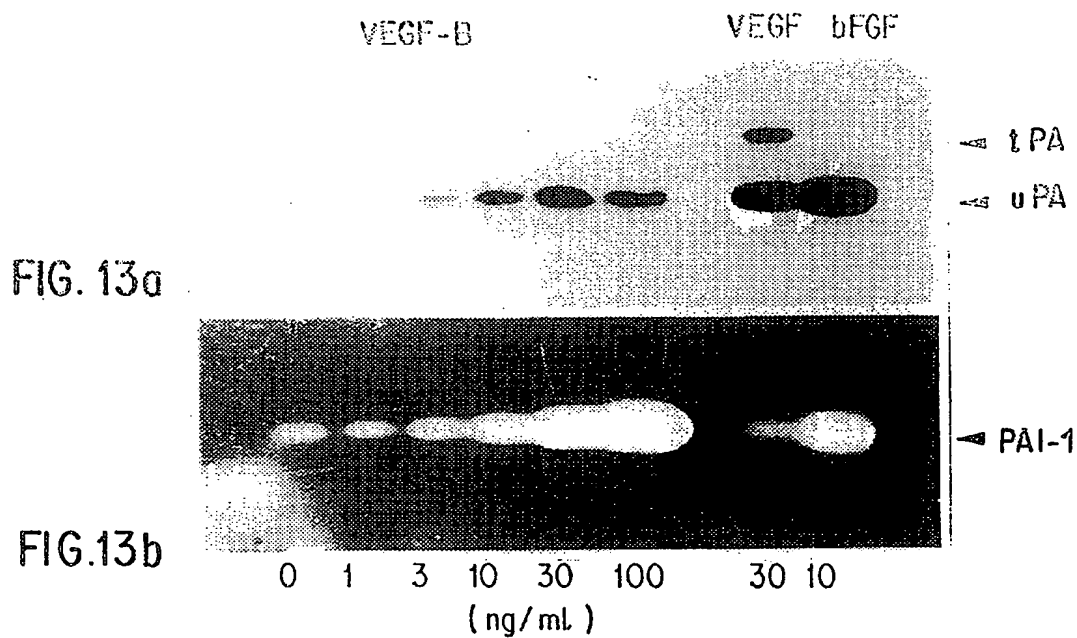


FIG.11



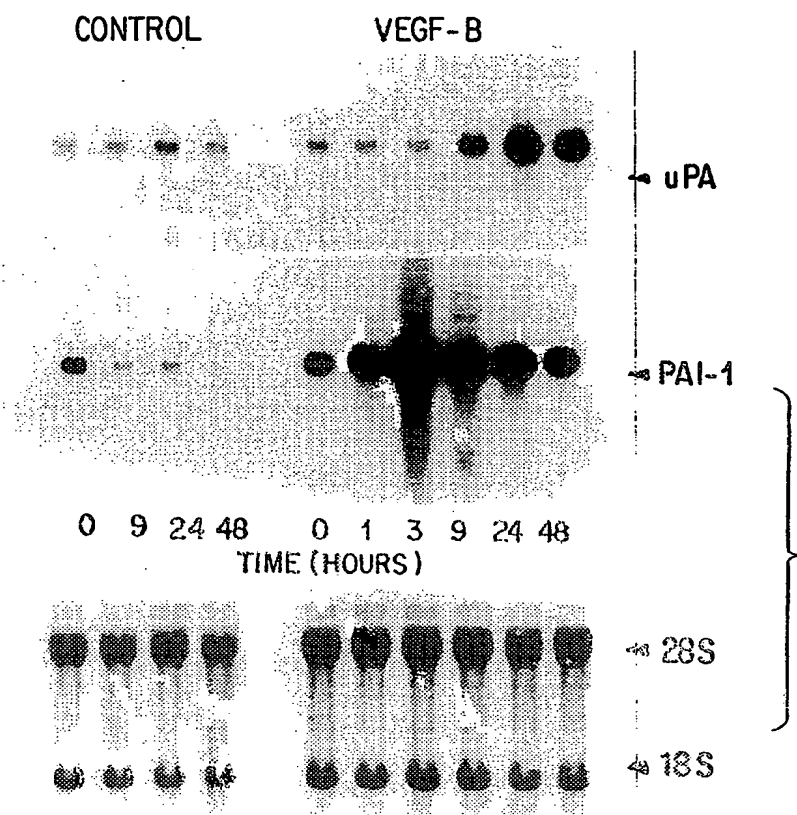


FIG. 12

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US97/23533

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 975; 530/350, 399, 387.9; 424/193.1, 158.1, 198.1; 514/2; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

search terms: flk-1, KDR, flt-1, VEGF, VPF, PIGF

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 5,712,395 A (APP et al.) 27 January 1998, entire document.	21,22
X ----- A	KEYT, B.A. et al., Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors J. Biol. Chem. 08 March 1996. Vol. 271. No.10. Pages 5638-5646, especially page 5639.	1-3, 5-10 ----- 18-21
X	LEE, J. et al. Vascular endothelial growth factor-related protein: A ligand and specific activator of the tyrosine kinase receptor Flt4. Proc. Nat. Acad. Sci. March 1996. Vol.93. pages 1988-1992, especially page 1990.	1, 5, 6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

*	Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A	document defining the general state of the art which is not considered to be of particular relevance		
B	earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means		
P	document published prior to the international filing date but later than the priority date claimed	*A*	document member of the same patent family

Date of the actual completion of the international search

30 APRIL 1998

Date of mailing of the international search report

03 JUN 1998

 Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23533

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- A	OLOFSSON, B. et al. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc. Nat. Acad. Sci. March 1996. Vol. 93 pages 2576-2581, especially page 2577.	2, 3 ----- 12
Y	SAWANO, A. et al. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular Endothelial Growth Factor 1. Cell Growth & Differentiation February 1996. Vol. 7. No. 2. pages 213-221, especially p. 217.	18
X ----- Y	WO 96/26736 A1 (LUGWIG INSTITUTE FOR CANCER RESEARCH.) 06 September 1996, entire document, especially pp. 31, 32, 43, 47, 48, 51.	2,3,11,13-17,22- 24,27 ----- 4,19-21,25,26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23533

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23533

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/53; A61K 39/385, 39/395, 38/18; C07H 21/04; C07K 14/71, 14/475, 16/28

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 975; 530/350, 399, 387.9; 424/193.1, 158.1, 198.1; 514/2; 536/23.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-10, drawn to a ligand-receptor complex of VEGF-B and Flt-1, a method of identifying VEGF-B analogs using Flt-1 polypeptide and a kit for use with the method.

Group II, claim(s) 11-15, drawn to a binding partner of the ligand-receptor complex.

Group III, claim(s) 16-21, drawn to a method of using a VEGF-B analog antagonist.

Group IV, claim(s) 22, drawn to a method of administering a VEGF-B agonist.

Group V, claim(s) 23-27, drawn to a VEGF-B analog and a polynucleotide encoding it.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is the ligand-receptor complex of VEGF-B and Flt-1. Group I also includes a method of using the receptor, Flt-1, to identify the ligand, VEGF-B, via a ligand-receptor complex.

The binding partner of Group II does not share the same special technical feature with Group I because the binding partner, such as an antibody, is structurally and functionally different from the ligand-receptor complex of Group I. In addition, the binding partner of Group II is not required for the identification method of Group I.

The antagonist of Group III does not share the same special technical feature with Group I because none of the antagonists are a ligand-receptor complex. Thus, the antagonists of Group III are structurally and functionally different from the ligand-receptor complex of Group I. Although the method of Group I may identify an antagonist of Group III, the antagonist of Group III is not required for the method of Group I.

The method of Group IV does not share the same special technical feature with Group I because the method does not use the ligand-receptor complex of Group I. Furthermore, the method has different process steps than the method of Group I, and is not required for the function of Group I.

The VEGF-B analog of Group V does not share the same special technical feature with Group I because the analog is structurally and functionally distinct from the ligand-receptor complex of Group I. Furthermore, the analog of Group V is not required for the identification method of Group I.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.